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Loss of Dimerisation of the Nonstructural Protein NS1 of Kunjin Virus Delays Viral Replication and Reduces Virulence in Mice, but Still Allows Secretion of NS1

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The flavivirus nonstructural protein NS1 has been implicated in viral RNA replication, although its precise role has not been identified. In its native state NS1 exists as a heat labile homodimer that is thought to be required for NS1 function and secretion. However, we have recently identified a cDNA clone of KUN virus (FLSD) that replicates efficiently in cell culture but produces and secretes NS1 in monomeric form. Sequence analysis of the NS1 gene in FLSD revealed a single amino acid substitution (proline^{250} to leucine) when compared with the parental KUN virus. When site-directed mutagenesis was used to substitute leucine^{250} with proline in FLSD to produce the clone 250pro, dimerisation was fully restored. Furthermore, time course experiments revealed that 250pro replicated in Vero cells significantly faster than FLSD and produced 100-fold more infectious virus early (12–24 h) in infection. This correlated with our observations that FLSD required approximately 10-fold more infectious virus than 250pro to produce disease in weanling mice after intraperitoneal inoculation. Taken together our results indicate that mutation from proline to leucine at residue 250 in KUN NS1 ablates dimer formation, slows virus replication, and reduces virulence in mice.

INTRODUCTION

The genus *Flavivirus* (family Flaviviridae) includes pathogens of global importance, such as yellow fever, Japanese encephalitis, and dengue viruses. There are approximately 70 members of this genus and most are transmitted by mosquitoes or ticks. Flaviviruses are small spherical viruses consisting of an icosohedral nucleocapsid surrounded by a lipid envelope embedded with the viral envelope proteins (reviewed by Rice, 1996). The flavivirus genome consists of a single strand of positive sense RNA approximately 11 kb in length. Two short untranslated regions at both the 5′ and 3′ termini flank a single open reading frame that codes for a polyprotein that is co- and posttranslationally cleaved to produce 3 structural (C, prM/M and E) and 7 nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5). The nonstructural protein NS1 is a 45K glycoprotein, which is expressed on the surface of and secreted from infected mammalian cells (Schlesinger *et al.,* 1990; Mason, 1989), and has been shown to induce protective immunity to flaviviruses in laboratory animals via antibody-mediated immune cytolysis (Schlesinger *et al.,* 1986, 1990; Timofeef *et al.,* 1998). Recent studies have shown that NS1 colocalises with double-stranded RNA and other replication-associated NS proteins in the infected cell (Mackenzie *et al.,* 1996; Westaway *et al.,* 1997) and is required for viral RNA replication (Muylaert *et al.,* 1997; Lindenbach and Rice, 1997). However, its precise role in this process is unclear. In its mature state, NS1 exists as a homodimer in cell-associated forms (Winkler *et al.,* 1988). In the extracellular fluid, hexameric species of NS1 are observed, apparently formed from dimeric subunits (Crooks *et al.,* 1994; Flamand *et al.,* 1999). The dimer is formed soon after NS1 is synthesised and translocated into the lumen of the ER, where it undergoes further processing (Winkler *et al.,* 1989; Mason, 1989). Based on the differential behaviour of monomeric versus dimeric NS1 in hydrophobicity studies it is possible that hydrophilic sites are buried inside the dimer, producing a more hydrophobic molecule (Winkler *et al.,* 1989). This would presumably give the protein the amphipathic qualities that allow it to exist in both membrane-associated and membrane-free states. Similarly, the incomplete conversion of a high mannose glycan to a complex glycan at the second conserved glycosylation site in secreted NS1 of both DEN 2 (Pryor and Wright, 1994) and MVE (B. Shiell, D. Scanlon, B. Blitvich, and R. Hall, unpublished results) indicates that this site is inaccessible in the dimer for modification during passage through the Golgi complex (Flamand *et al.,* 1992).

Previous studies performing site-directed mutagenesis on the NS1 gene to destabilise dimer formation in both transiently expressed protein and flavivirus infectious clones have suggested that dimerisation is re-

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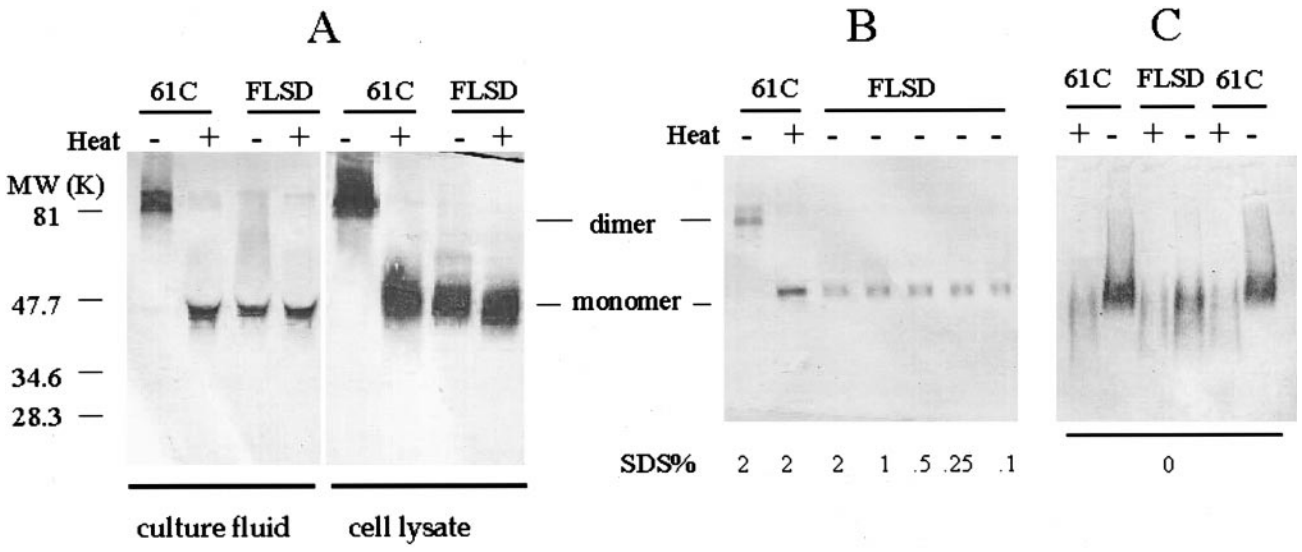


FIG. 1. Western blot analysis of NS1 dimer formation by KUN viruses MRM61C and FLSD. (A) Culture fluids and cell lysates of MRM61C- and FLSD-infected Vero cultures were boiled or unheated in sample buffer and proteins separated by 10% SDS–PAGE, electroblotted onto nitrocellulose membranes, and immunostained with anti-NS1 Mab. (B) FLSD-infected culture fluid was exposed to a range of SDS concentrations in the sample buffer without heating and NS1 dimer formation analysed by SDS–PAGE and Western blot. (C) MRM61C- and FLSD-infected culture fluids were boiled or unheated in the absence of SDS and NS1 dimer formation was analysed by native PAGE and Western blot.

quired for NS1 secretion and virus replication (Pryor and Wright, 1993, 1994; Pryor *et al.,* 1998). However, in this paper we show that substitution of a conserved proline residue in the NS1 protein of Kunjin virus results in loss of dimer formation, but permits secretion of the protein and allows efficient virus replication. We also report the effect of this mutation on viral growth kinetics in cell culture and on virulence in mice.

RESULTS

A clone of KUN virus produces NS1 as a monomer

When we examined culture fluid or lysates of Vero cells infected with the KUN strain MRM61C, NS1 appeared in Western blots as a 90K band in unheated samples which converted to 45K when samples were boiled (Fig. 1A). This was consistent with previous findings that the flavivirus NS1 protein existed as a heat labile homodimer (Winkler *et al.,* 1988). In contrast, the NS1 protein of the FLSD virus generated from one of the full-length KUN cDNA clones (Khromykh *et al.,* 1998) was detected as a 45K protein in both boiled and unheated samples (Fig. 1A). This indicated that NS1 of FLSD virus was detected in monomer form even without exposure to heat denaturing conditions. In previous studies we have observed some antigenic domains of flaviviral proteins to be highly sensitive to even trace amounts of the strong ionic detergent SDS (Hall *et al.,* 1990; R. Hall, unpublished results). Therefore, to assess whether exposure to detergent was responsible for the disruption of the NS1 dimer in FLSD during PAGE analysis, the sensitivity to SDS was determined by exposing the protein to various concentrations (0.1, 0.25, 0.5, 1, and 2%) in the sample buffer without heating, prior to electrophoresis. However, even at the lowest SDS concentration, NS1 was observed in monomer form (Fig. 1B). We then subjected the samples to PAGE in the absence of SDS (native PAGE), prior to transfer to membranes and immunoblotting. While NS1 appeared as poorly resolved smears in these blots due to both charge and molecular mass influencing protein mobility in the absence of SDS, it was clear that the unheated form of NS1 of MRM61C virus displayed a significantly slower mobility than the boiled form (Fig. 1C). This was consistent with the multimeric structure of the former and the monomeric structure of the latter. On the other hand the NS1 protein in unheated FLSD samples showed a migration pattern similar to that of the boiled protein. This indicates that the NS1 protein of KUN clone FLSD exists in monomeric form under native conditions.

A single amino acid substitution in the NS1 protein of KUN virus ablates dimer formation, but allows its secretion

To determine the cause of loss of dimer formation by the FLSD virus we sequenced the entire NS1 gene of this clone and compared the translated amino acid sequence with the published sequence of the MRM61C parental strain (Coia *et al.,* 1988). Three amino acid substitutions were observed: $Ile^{29} \rightarrow Met$, $Pro^{250} \rightarrow Leu$, and $Asn^{152} \rightarrow Ser$ (Fig. 2). To confirm that these changes were unique to the FLSD clone we also sequenced the corresponding regions in the genome of our lab stocks of MRM61C and

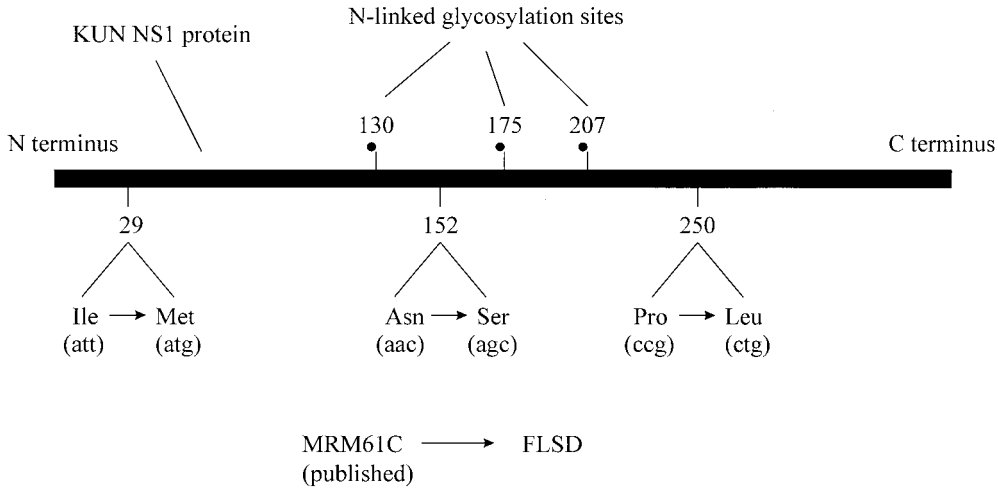


FIG. 2. Schematic representation of the NS1 protein of KUN virus showing amino acid changes between FLSD and the published sequence of MRM61C (Coia *et al.,* 1988). The three glycosylation sites are also marked for reference.

another KUN virus, OR393. The results revealed that Pro^{250} → Leu was unique to FLSD and that MRM61C and OR393 both possessed a proline at this position (results not shown). However, our lab stocks of the MRM61C and OR393 KUN strains were also shown to have Met at residue 29 and a Ser at residue 152, indicating that the original published sequence of MRM61C showing Ile and Asn at these positions may have been incorrect, possibly due to mutations that occurred during cloning. In summary, these results revealed that a single amino acid substitution (Pro^{250} → Leu) in the NS1 protein of FLSD resulted in loss of NS1 dimerisation.

To confirm that the loss of dimerisation of NS1 in the FLSD virus was indeed due to the observed amino acid substitution in NS1 and not to an unidentified mutation in other viral proteins that may interact with NS1 (Blitvich *et al.,* 1995; Lindenbach and Rice, 1999) and therefore indirectly affect dimerisation, we used site-directed mutagenesis to replace Leu with Pro at position 250 in NS1 of FLSD to restore it to the wild-type sequence. The resulting cDNA clone was designated p250pro. When culture fluids from Vero cells infected with the 250pro virus derived from the p250pro cDNA clone were analysed by Western blot, dimerisation of NS1 was found to be completely restored, showing identical mobility in SDS–PAGE to NS1 of MRM61C virus (Fig. 3A). This conclusively demonstrated that the substitution of Pro^{250} to Leu in NS1 of FLSD was responsible for the loss of dimer formation.

Previous studies have suggested that dimerisation was required for efficient secretion of NS1 from flavivirus-infected mammalian cells. Therefore, the efficiency of NS1 secretion from FLSD-infected Vero cells was assessed by Western blot and compared to that of

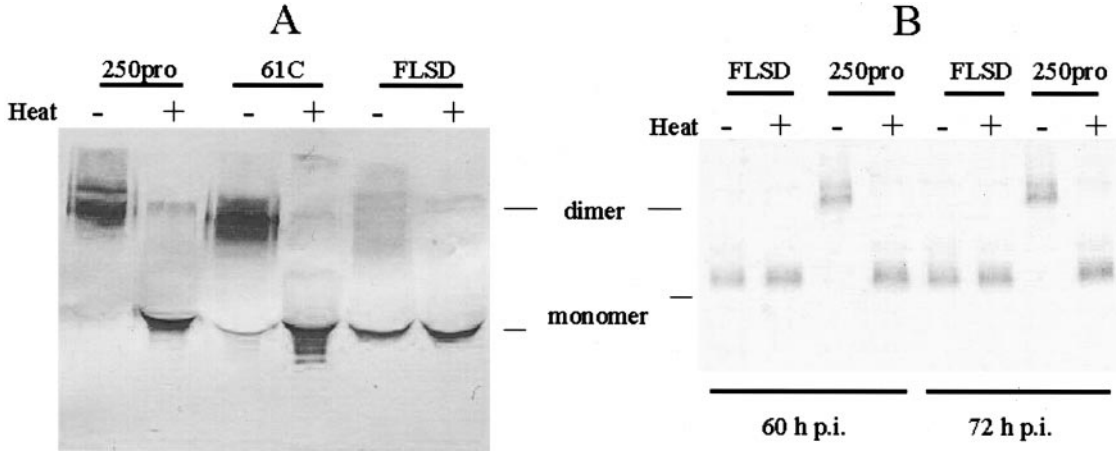


FIG. 3. Substitution of Pro for Leu at residue 250 in NS1 of FLSD fully restores dimerisation. (A) Culture fluids were harvested from 250pro-, MRM61C-, and FLSD-infected Vero cells at approximately 96 h p.i., boiled or unheated in sample buffer, and analysed for NS1 dimer formation by SDS–PAGE and Western blot. (B) Culture fluids from Vero cells infected with FLSD or 250pro viruses at an m.o.i. of 0.1 were harvested at 60 or 72 h p.i., boiled or unheated, and analysed for the presence of NS1 by SDS–PAGE and Western blot.

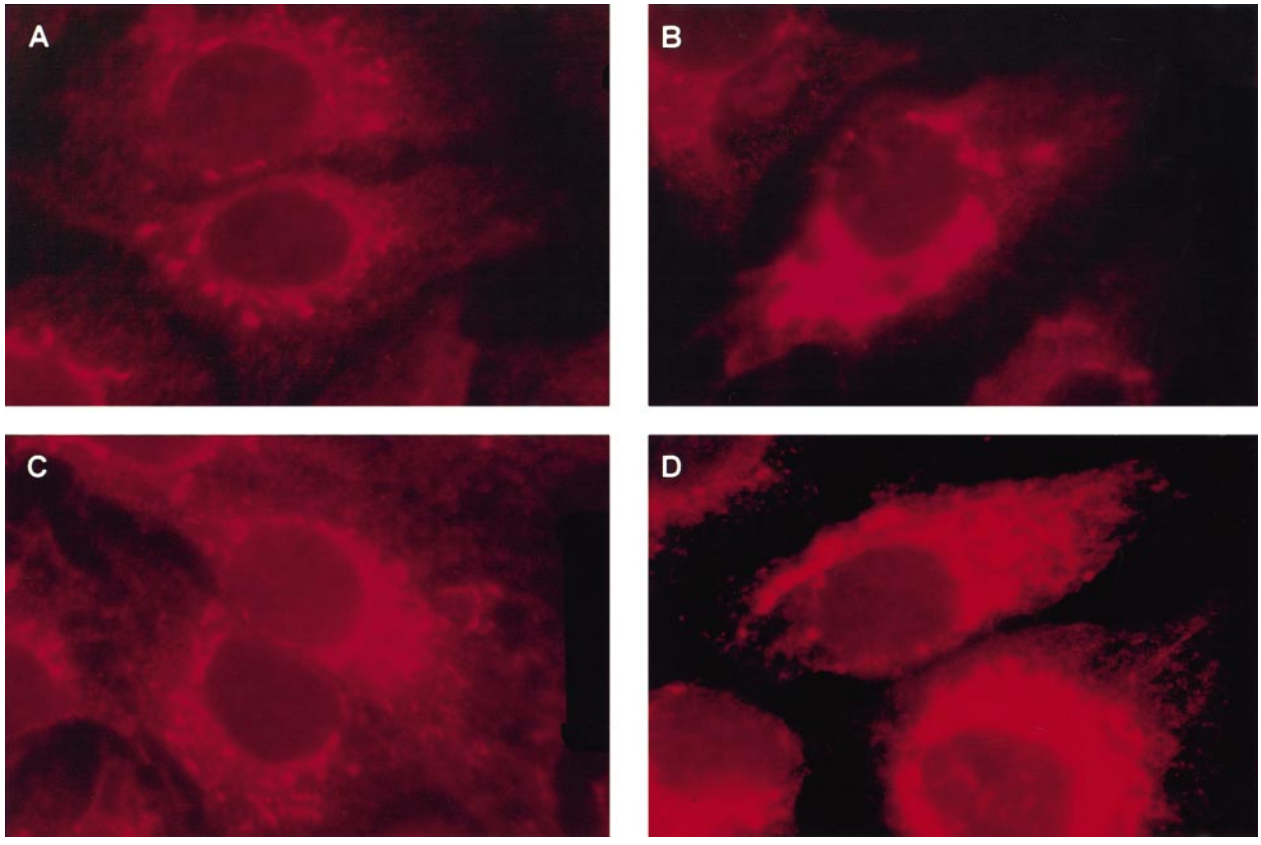


FIG. 4. Immunoflouresence of NS1 in FLSD- and 250pro-infected cells. Vero cells were grown on coverslips and infected at an m.o.i. of 2 with 250pro (A and B) or FLSD (C and D) for 24 h p.i. before fixation with acetone at −20°C for 30 s. Coverslips were then probed with anti-NS1 rabbit antiserum and species-specific Texas red conjugate (Edward Keller, Australia) as previously described (Westaway *et al.,* 1997).

250pro-infected cultures. The relative intensity of the NS1 band in culture supernatant harvested at 60 and 72 h postinfection (p.i.) indicated that NS1 was present in the FLSD samples at levels similar to those of the 250pro samples (Fig. 3B). This demonstrates that monomeric NS1 produced by FLSD virus is secreted with similar efficiency to the dimeric NS1 produced by 250pro.

To assess whether monomeric NS1 displayed an intracellular distribution similar to that of the dimeric form, immunofluoresence was performed on Vero cells infected for 24 h with the FLSD and 250pro viruses using NS1-specific rabbit serum. As can be observed in Fig. 4, the distribution of NS1 produced by the 250pro (Figs. 4A and 4B) and FLSD (C and D) viruses appeared similar. NS1 displayed two staining patterns which appeared as either a thickened perinuclear reticular staining, at times including isolated cytoplasmic foci (Figs. 4B and 4D), analogous to that observed after 24 h p.i. with wild-type KUN (Westaway *et al.,* 1997), or composed mainly of small aggregated foci in the perinuclear region, with some faint reticular staining in the cytoplasm. Small cytoplasmic foci were also occasionally seen (Figs. 4A and 4C). This latter NS1 distribution is similar to the staining pattern observed at 16 h p.i. with wild-type KUN (Westaway *et al.,* 1997) and therefore may represent individual cells at different stages of infection. In summary, the results presented suggest that the overall distributions of NS1 from FLSD and 250pro appear similar and that the staining patterns observed are comparable to those patterns observed during infection of Vero cells with wild-type KUN.

Loss of NS1 dimer formation in FLSD is associated with delayed virus replication in cell culture and reduced virulence in mice

To assess the effect of this structural change in NS1 on viral replication we investigated the growth kinetics of FLSD virus in cell culture. Vero monolayers were infected with FLSD, 250pro, or MRM61C viruses at an m.o.i. of 0.1 or 1, and culture fluids assayed for infectious virus titre at regular intervals between 0 and 48 h postinfection. The results revealed that after infection at an m.o.i. of 0.1, virus was first detected in the supernatant of each culture at 18 h p.i. (Fig. 5A). At this time point culture fluid from 250pro-infected cells contained almost 100 times more infectious virus than FLSD-infected culture fluid ($10^{4.7}$ and $10^{2.9}$ infectious units/ml, respectively) and continued to show at least 10-fold more virus in samples taken up to 42 h p.i. In comparison, MRM61C-infected

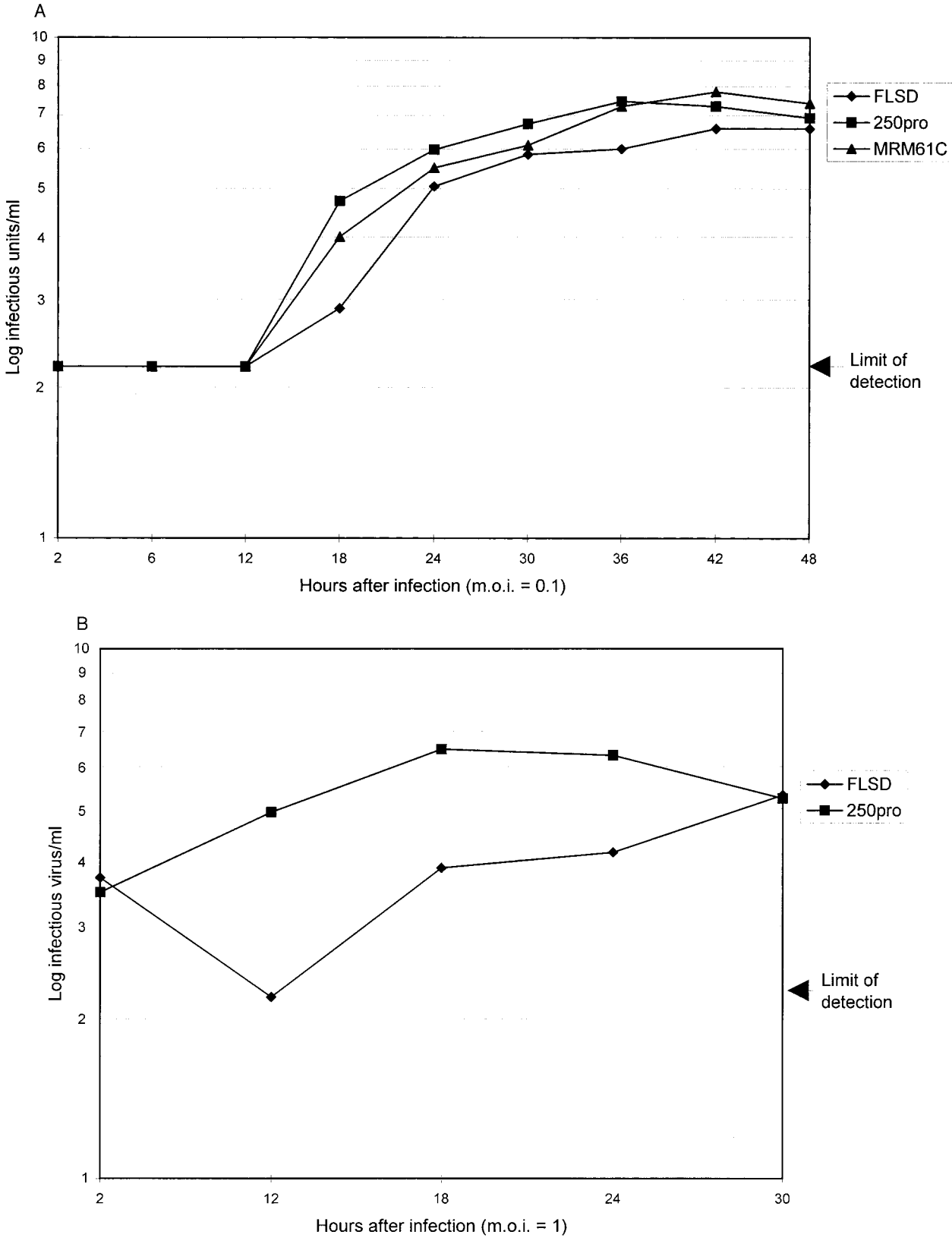


FIG. 5. Kinetics of viral replication in FLSD-infected cultures. (A) Vero cultures were inoculated with FLSD, 250pro, or MRM61C viruses at an m.o.i. of 0.1 or (B) with FLSD or 250pro at an m.o.i. of 1, and culture fluid was collected at regular intervals between 0 and 48 h p.i. and analysed for infectious viral titre by $TCID_{50}$ assay. Each graph represents the results of a single experiment.

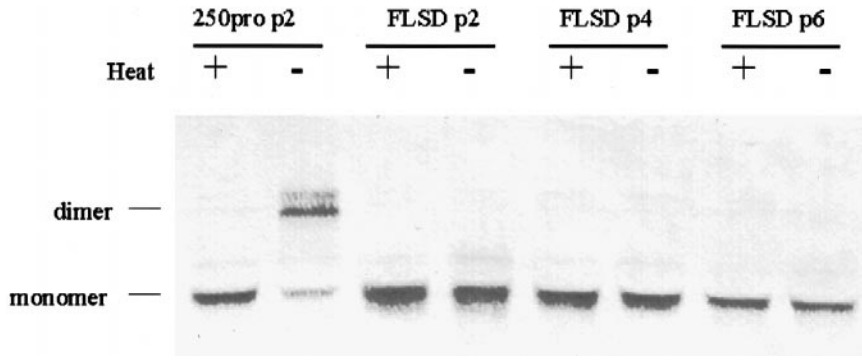


FIG. 6. Stability of the monomeric NS1 phenotype of FLSD during serial passage in Vero cells. FLSD virus produced from RNA-transfected BHK cells was serially passaged six times in Vero cells, and culture fluids from passage 2 (p2), 4 (p4), and 6 (p6) were analysed by Western blot for NS1 dimer formation, as previously described. Samples of 250pro at passage 2 were similarly analysed for reference.

culture fluids contained slightly lower or similar titres to 250pro at these times. The titres for all three viruses were similar at 48 h. When Vero cultures were infected at an m.o.i. of 1, progeny virus (ignoring the residual inoculum detected 2 h after infection) was detected in 250pro culture fluid by 12 h p.i., but no virus was detected in FLSD culture fluid until 18 h p.i. (Fig. 5B). Again, the titres for 250pro culture fluids were at least 100-fold greater than those for FLSD virus between 12 and 24 h, while titres became similar later in infection. In summary, 250pro virus shows growth kinetics similar to those of MRM61C in Vero cells, but grows significantly faster than FLSD virus early in infection.

Since only a single base change (ctg to ccg) in the codon corresponding to residue 250 in FLSD RNA is required during viral replication to produce a dimeric NS1 phenotype, we examined the stability of FLSD during serial passage in Vero cells. No dimeric NS1 was detected even after six passages of FLSD virus in Vero cells (Fig. 6), thus demonstrating stable retention of $Pro^{250} \rightarrow$ Leu mutation in FLSD viral RNA during passaging.

To determine the effect of the mutation in NS1 on the virulence of FLSD, 18- to 20-day-old weanling BALB/c mice were inoculated i.p. with 10-fold dilutions of FLSD and 250pro viruses. The results show that FLSD required approximately 10-fold more infectious units (as determined by titration in Vero cells) to produce disease than 250pro, with one LD_{50} corresponding to approximately 2000 and 200 infectious units for FLSD and 250pro viruses, respectively (Fig. 7).

DISCUSSION

In this paper we present the first compelling evidence that dimerisation of the flavivirus NS1 protein is not required for virus replication and for secretion of the NS1 protein from infected cells or for virus replication and neurovirulence in mice. Since the discovery by Winkler *et al.* (1988) that the NS1 protein of the flaviviruses DEN 2, St. Louis encephalitis, and Powassan encephalitis existed as a heat labile homodimer, several studies have confirmed that dimerisation of NS1 is indeed a general feature of flaviviruses (Fan and Mason, 1990; Crooks *et al.,* 1994; Hall *et al.,* 1990; Hall and Pomery, unpublished results). This strict conservation of structure suggested that dimerisation was functionally important to NS1 and may be required to facilitate its proposed role in viral RNA replication (Mackenzie *et al.,* 1996; Muylaert *et al.,* 1997; Lindenbach and Rice, 1997; Westaway *et al.,* 1997). This hypothesis was supported by Pryor and Wright (1993, 1994), who demonstrated that alanine substitutions for selected cysteines, glycosylated asparagines, or hydrophilic residues in DEN 2 NS1 expressed by a mammalian expression vector resulted in loss of dimerisation and severely effected the normal cellular processing and secretion of the protein. However, when the substitutions that completely abolished dimerisation in two transiently expressed mutants of NS1 ($Glu^{173} \rightarrow$ Ala and $Lys^{174} \rightarrow$ Ala; and $Asn^{207} \rightarrow$ Ala) were introduced into a full-length cDNA clone of DEN 2, they failed to significantly effect NS1 dimerisation in cells infected with recovered viruses, suggesting that dimerisation studies on transiently expressed protein were not indicative of protein structure during viral infection (Pryor *et al.,* 1998). Indeed, our finding that KUN clone FLSD replicates efficiently in cell culture while producing NS1 in monomer form indicates that dimerisation is not an absolute requirement for NS1 function. The apparently normal intracellular distribution of the protein in FLSD-infected cells and the relatively efficient secretion of NS1 observed in these cultures also suggest that the intracellular trafficking of monomeric NS1 is not significantly affected.

A significant delay in virus production was observed during the first 12–24 h of FLSD infection in Vero cells, suggesting that the monomeric NS1 produced by FLSD virus is less efficient in replication than wild-type NS1 during the early stages of infection. This is consistent with a proposed involvement of NS1 during the early

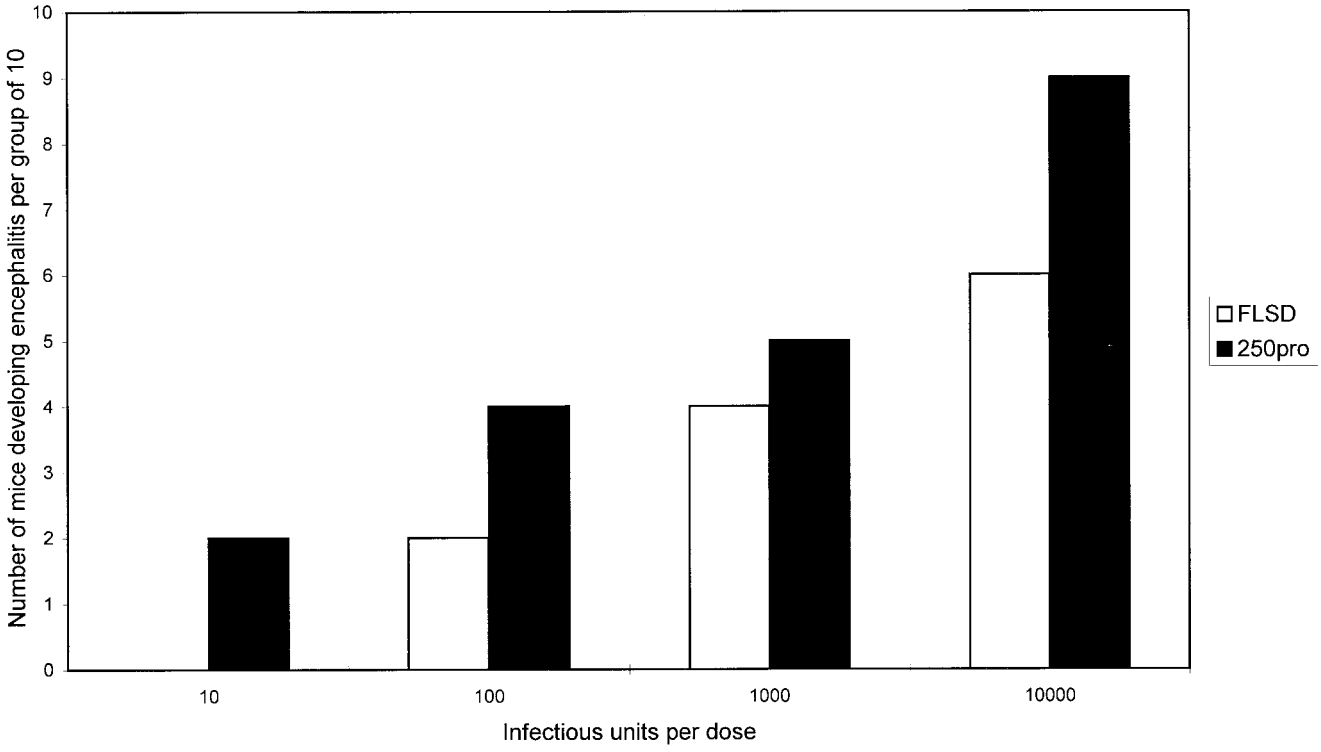


FIG. 7. Virulence of FLSD and 250pro in 18- to 20-day-old mice. Groups of 10 18- to 20-day-old BALB/c mice were inoculated i.p. with a range of doses of FLSD or 250pro viruses. The bar graph represents the number of mice developing encephalitic symptoms per group.

phase of viral RNA replication (Lindenbach and Rice, 1997). The fact that viral titers reached similar levels by 30–48 h postinfection (depending on the m.o.i.) indicates that later stages of virus replication were not affected. Analysis of the accumulation of negative versus positive-strand viral RNAs in FLSD- and 250pro-infected cultures will clarify this issue and may provide further evidence for a precise role of NS1 in RNA replication.

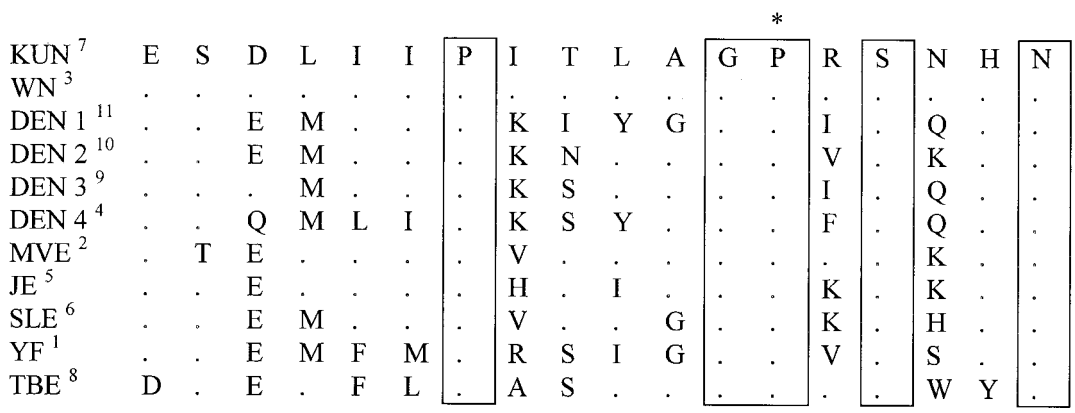
Our studies on the relative virulence of FLSD and 250pro viruses in weanling mice after intraperitoneal (i.p.) inoculation revealed that FLSD required approximately 10-fold more virus to produce disease. Combined with the results showing delayed replication of FLSD virus in Vero cell cultures, the *in vivo* studies indicate that a lag in virus production during the early stages of replication probably results in less efficient invasion of the central nervous system before the virus is cleared by the immune response.

Thus we found that a single amino acid substitution (Pro → Leu) at position 250 in the NS1 protein of Kunjin virus was responsible for loss of NS1 dimerisation, slower virus growth, and diminished virulence. Although this substitution may be considered conservative (since both amino acids are hydrophobic), Pro is quite unique amongst the amino acids in that the last carbon atom of the side chain is bonded to the main chain nitrogen atom forming a ring structure. Pro is often found in flexible regions of a polypeptide or provides a bend in the peptide chain (Branden and Tooze, 1991). A substitution of Pro to Leu is likely to have a significant effect on the structure of the surrounding peptide sequence and is consistent with the major structural change indicated by the lack of dimerisation of the mutated NS1. Indeed, a search of the databases revealed that the Pro at position 250 in NS1 and a second Pro, six residues upstream, are strictly conserved in all members of the flavivirus genus sequenced to date, and both lie in a relatively conserved region of the protein (Fig. 8). This suggests that this stretch of amino acids is critical for NS1 dimerisation and that similar mutations in other flaviviruses may also effect the dimerisation of NS1, replication efficiency, and virulence. We are currently investigating this using infectious cDNA clones of other flaviviruses.

MATERIALS AND METHODS

Cell and virus culture

Vero cells were grown in HEPES-buffered Medium 199 supplemented with antibiotics and 10% FBS and incubated at 37°C. For virus stock production, Vero cells were infected with wild-type KUN strains MRM61C or OR393 (Adams *et al.,* 1995) or virus derived from KUN cDNA clones (Khromykh and Westaway, 1994; Khromykh *et al.,* 1998) at an m.o.i. of 0.1–1 and cultured in medium supplemented with 2% FBS. Culture supernatant was harvested and clarified at 72–96 h postinfection when 50–70% of cells were showing CPE.



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KUN [7]	E	S	D	L	I	I	P	I	T	L	A	G	P	R	S	N	H	N
WN [3]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DEN 1 [11]	.	.	E	M	.	.	.	K	I	Y	G	.	.	I	.	Q	.	.
DEN 2 [10]	.	.	E	M	.	.	.	K	N	.	.	.	.	V	.	K	.	.
DEN 3 [9]	.	.	.	M	.	.	.	K	S	.	.	.	.	I	.	Q	.	.
DEN 4 [4]	.	.	Q	M	L	I	.	K	S	Y	.	.	.	F	.	Q	.	.
MVE [2]	.	T	E	.	.	.	.	V	.	.	.	.	.	.	.	K	.	.
JE [5]	.	.	E	.	.	.	.	H	.	I	.	.	.	K	.	K	.	.
SLE [6]	.	.	E	M	.	.	.	V	.	.	G	.	.	K	.	H	.	.
YF [1]	.	.	E	M	F	M	.	R	S	I	G	.	.	V	.	S	.	.
TBE [8]	D	.	E	.	F	L	.	A	S	.	.	.	.	.	.	W	Y	.

FIG. 8. Pro[250] in NS1 is strictly conserved in all *Flavivirus* species. Alignment of the Kunjin amino acid sequence spanning the Pro at residue 250 (denoted by *) in NS1 with the corresponding sequence of other flaviviruses ([1]Rice *et al.,* 1985; [2]Dalgarno *et al.,* 1986; [3]Castle *et al.,* 1986; [4]Mackow *et al.,* 1987; [5]Sumiyoshi *et al.,* 1987; [6]Trent *et al.,* 1987; [7]Coia *et al.,* 1988; [8]Mandl *et al.,* 1989; [9]Osatomi and Sumiyoshi, 1990; [10]Blok *et al.,* 1992; [11]Fu *et al.,* 1992). Dots represent amino acid identity and boxed residues are conserved in all flaviviruses.

PAGE and Western blots

Samples were prepared as cell lysates or culture supernatants and analysed by nonreducing SDS–PAGE on 7.5 or 10% gels as previously described (Adams *et al.,* 1995). For native PAGE, 5× sample buffer without SDS was diluted in virus culture supernatant and samples were electrophoresed on 7.5% polyacrylamide gels without SDS for 3–4 h at 100 V in the cold. SDS was also omitted from the running buffer, which was buffered to pH 8.3. Electrophoresed proteins in SDS and native gels were electroblotted onto nitocellulose membranes (Hybond C, Amersham) and immunostained with a cocktail of KUN NS1-reactive monoclonal antibodies as previously described (Adams *et al.,* 1995; Blitvich *et al.,* 1995).

Viral RNA extraction, RT/PCR, and DNA sequencing

Infected Vero cell monolayers in 25-cm^2 flasks were drained of culture medium and lysed in Total RNA Isolation Reagent (Advanced Biotechnologies) and RNA was extracted according to the manufacturer's instructions. One to 2 μl of the RNA preparation was then used as template in an RT/PCR using a previously described procedure (Poidinger *et al.,* 1996). PCR products or plasmids were sequenced using BIG-DYE chemistry (ABI) and an ABI automated sequencer according to the manufacturer's instructions.

Site-directed mutagenesis

The construction of plasmid FLSD, which contains a full-length cDNA copy of the KUN virus genome in a pBR322 backbone, has previously been described (Khromykh and Westaway, 1994; Khromykh *et al.,* 1998). To substitute the leucine with proline at amino acid residue 250 in NS1 of KUN FLSD, primers 5843s (CCTCCGTGGGAAAGGCTGTCC-forward) and 7404 (TTCTTGGTTGGTCCATCTCGC-reverse) were designed to flank the *Sph*I sites at nucleotide positions 6038 and 7198 in the plasmid. The mutagenesis primer pro250ΔNheI (GCTTCGCGGTCCCGCAAGCG) and its complement pro250ΔNheIcomp were designed to substitute leucine 250 with proline and to eliminate the *Nhe*I site at position 6778 in the plasmid. PCR products were then generated from pFLSD template using primer pairs (5843s-p250ΔNheIcomp) and (p250ΔNheI-7404) and the high-fidelity polymerase PFU (Stratagene). These PCR products were then gel purified and used as overlapping templates with the primer pair 5843s and 7404 in a PCR reaction to produce a 1.6-kb fragment spanning the aforementioned *Sph*I sites and containing the desired mutations. This fragment was digested with *Sph*I and ligated into *Sph*I-digested and dephosphorylated pFLSD. The correct orientation of the insert in the resulting clones was identified by colony PCR and the presence of the mutations was confirmed by digesting miniprep DNA with *Nhe*I and sequencing selected clones. The resulting plasmid containing the desired mutations was designated p250pro and three separate clones were selected for preparation of RNA for transfections.

Viral RNA transcription and transfection

RNA transcripts were prepared from *Xho*I-linearised plasmids (pFLSD and p250pro) using SP6 RNA polymerase as previously described (Khromykh and Westaway, 1994; Khromykh *et al.,* 1998). Approximately 5–10 μg of *in vitro* transcribed RNA was electroporated into 2×10^6 BHK21 cells in 400 μl in a 0.2-cm electrode gap cuvette with a Gene Pulser apparatus (Bio-Rad) at 1.5 kV, 25 μF, and ∞ resistance. Transfected cells were incubated at 37°C in DMEM with 10% FBS, and culture supernatant was harvested for stock virus when CPE was observed.

Growth kinetics of virus in Vero cells

Monolayers of Vero cells in 1.5-cm-diameter culture wells were infected with an m.o.i. of 0.1 or 1 with FLSD,

250pro, or MRM61C viruses in 200 μl. After 2 h the inoculum was removed and wells were rinsed gently with 1 ml of fresh medium. Wells were then replenished with 1 ml of fresh medium containing 2% FBS. Samples of culture medium were harvested from infected wells at 6-h intervals between 0 and 48 h p.i. and stored at −70°C until tested for infectious viral titre. To determine the infectious viral titre in each sample, 10-fold dilutions of the virus were inoculated onto Vero cells in 96-well plates, using 10 wells per dilution, and plates incubated for 7 days and then examined microscopically for CPE. The $TCID_{50}$ titre was calculated using the method of Reed and Meunch (1938).

Mouse virulence studies

BALB/c mice between 18 and 20 days of age were inoculated in groups of 10 by the i.p. route with 100 μl containing 10, 100, 1000, or 10,000 infectious units of either FLSD or 250pro virus. Mice were closely monitored for 21 days and were culled when signs of paralysis or encephalitis were evident. The LD_{50} for each virus was calculated using the method of Reed and Meunch (1938). These experiments were conducted with approval from the University of Queensland Animal Experimentation Ethics Committee in accordance with the guidelines for animal experimentation as set out by the National Health and Medical Research Council, Australia.

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